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Microsatellite (SSR) variation in a collection of *Malus* (apple) species and hybrids

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Summary

A collection of 142 accessions of 23 *Malus* species, derived hybrids and cultivar accessions from the USDA-ARS Plant Genetic Resources Unit's core collection, which represents an extensive range of *Malus* species, was screened with a set of previously described SSR (simple sequence repeat) markers. The markers were used to determine genetic identities, estimate genetic diversity, identify genetic relationships among the accessions, and determine the utility of SSR primers developed from *Malus* × *domestica* for making genetic assessments across the whole *Malus* genus. All eight primer pairs amplified multiple fragments when used in polymerase chain reactions with DNA from these accessions. High levels of variation were detected with a mean of 26.4 alleles per locus and a mean direct count heterozygosity across all eight loci equal to 0.623. The eight primer pairs used in this study unambiguously differentiated all but five pairs of accessions in this collection of 142 accessions of 23 *Malus* species, derived hybrids and cultivars. These SSR data were not useful in identifying genetic relationships among this diverse collection of accessions, with the majority of the accessions not clustering in ways concordant with taxonomic information and/or geographic origin. The resulting phenogram resolved only two meaningful clusters, for the taxonomically isolated Section Chloromeles and for *M. fusca* accessions, reflecting genetic relationships arising from geographic origin. The detection of identical accessions in the collection, which were previously considered to be unique, highlights the critical need to further bolster collections of certain *Malus* species.

Introduction

The domesticated apple, *Malus* × *domestica* Borkh., is a complex hybrid of several *Malus* species including; *M. sieversii* (Ledeb.) M. Roemer, *M. orientalis* Uglitzk, *M. sylvestris* Miller, *M. baccata* (L). Borkh., *M. mandshurica* (Maxim) V. Komarov, and *M. prunifolia* (Willd.) Borkh. (Hokanson et al., 1997; Janick et al., 1996; Way et al., 1991). It is one of the most widely cultivated temperate fruit crops, produced commercially from Siberia and northern China, with winter temperatures ranging to –40 °C, to high elevation equatorial locations in Colombia and Indonesia where two crops can be produced in a single year (Janick, 1974). Woridwide apple production has more than doubled since 1970, from ∼21 to ∼55 million tons in 1997 (FAO, 1997).

The genetic base of domesticated apple has eroded dramatically. At one time over 7000 cultivars were described in the literature (1804–1904) (Ragan, 1926); now most of the world's production is based on only two cultivars: 'Delicious' and its red sports, and 'Golden Delicious'. Furthermore, present expansion is based largely on their seedlings: 'Gala', 'Mutsu', 'Jonagold', from 'Golden Delicious'; and 'Empire' and 'Fuji' from 'Delicious' (Janick et al., 1996). Exacerbating this decline in genetic diversity is a reduction in the number of breeding programs (Brooks & Vest, 1985; Frey, 1996). These declines have occurred in spite of the steady incursion of new insect and disease problems and the growing worldwide demand for improved apple cultivars with higher quality and resistance to biotic and abiotic stresses.

The genetic base of apple and the pool of traits and characters available for breeders to incorporate into domesticated apple would certainly be expanded by including wild *Malus* species in cultivar development programs. The genus *Malus* is variously reported to consist of 25–35 species, with the number still a matter of some debate and dependent on the authority (Rehder, 1940; Huckins, 1972; Watkins, 1981; Way et al., 1991; Langenfeld, 1991; Li, 1996). Species within the genus are widely distributed, although generally they are found in the northern temperate zones of North America, Europe, Asia Minor and Asia. One species, *M. doumeri* (Bois.) A. Chev., is found in more tropical regions of Taiwan. Such wide geographic distribution suggests that a wealth of potentially useful traits may exist within the genus that could be utilized by apple breeders in the development of modern apple cultivars and apple rootstocks suited to diverse environmental conditions.

Fertile hybrids are obtained from almost all crosses among *Malus* species, (Korban, 1986) and there is ample precedent for significant contributions by species for the improvement of commercial apple cultivars. Apple scab (*Venturia inaequalis* (Cke.) Wint.) is generally the most significant fungal disease of apple, and building resistance to the pathogen is an objective of most breeding programs in the world today. Nearly all the apple cultivars released since 1970 that are resistant to apple scab derive their resistance from *M.* × *floribunda* ‘821’ Siebold ex Van Houte (V_f), (Hough et al., 1953) although other scab resistance genes have been moved into commercial cultivars from *M. baccata* ‘Hansen’s 2’ (V_b), (Dayton & Williams, 1968; Williams & Kuc, 1969), *M. baccata* ‘Jackii Dg27T1’ (V_{bj}) (Dayton & Williams, 1968), *M. micromalus* Makino ‘245-38’ (V_m), (Dayton & Williams, 1970) and *M.* ‘Russian seedling’ (V_r) (Dayton & Williams, 1968). A partial listing of other disease resistances captured from *Malus* species includes resistance to powdery mildew (*Podosphaera leucotricha* (Ell. & Ev.) Salm.) derived from *M.* × *zumi* (Matsum.) Rehder and *M.* × *robusta* (Carriere) Rehder among others (Knight & Alston, 1968), and resistance to fireblight (*Erwinia amylovora* (Burr.) Winslow et al.) from a number of species and interspecific hybrids, including *M.* × *robusta* ‘No. 5’ and *M.* × *sublobata* (Dipp.) Rehder PI286613 (Gardner et al., 1980; van der Zwet & Keil, 1979).

Cold hardiness has been incorporated into domesticated apples using *M. baccata* and the closely-related *M. prunifolia*, as well as *M. sieversii*, which many consider the primary progenitor of modern cultivated apple (Stepanov, 1974; Strang & Stushnoff, 1975). In fact, recent collection trips to the center of diversity for *M. sieversii* in the Tien Shan mountain region of Kazakstan have encountered numerous genotypes with large, high quality fruits (Hokanson et al., 1997). Material collected in that region is being screened for resistance to apple scab (Aldwinckle et al., 1997), fireblight (Momol et al., 1999) and cedar apple rust, along with evaluations for general horticultural characters. Numerous other traits have been derived from various species including various insect resistances (Briggs & Alston, 1969; Brown et al., 1988; Dabrowski & Rejman, 1975; Goonewardene, 1987; Goonewardene & Howard, 1989), reduced juvenility (Watkins, 1973), dwarfing (Lapkins, 1969; Lapkins, 1976; Meulenbroek et al., 1999), and various rootstock characteristics (Cummins & Aldwinckle, 1980, 1983a, 1983b).

Nonetheless, the vast potential for genetic improvement in *Malus* lies virtually untapped. One impediment to a more systematic use of species material in apple cultivar development is a lack of information regarding traits and characters in the germplasm. To facilitate an improved characterization of the large USDA-ARS *Malus* germplasm collection maintained at the Plant Genetic Resources Unit (PGRU), a core subset collection was developed (Kresovich et al., 1995; Forsline, 1996). The core subset, selected to represent the diversity found within the entire collection (Frankel, 1984; Brown, 1989a; Marshall, 1990; Brown, 1995), has been established in a national, multi-site field replication to evaluate general and regionally important horticultural traits, biotic and abiotic resistances in several environments (Forsline, 1996; Forsline, 2000).

As one stage in the process of characterizing the Malus genome, we reported on the evaluation of a collection of 66 *Malus* × *domestica* accessions from the core collection held at the PGRU in Geneva, New York (Hokanson et al., 1998). In that study we used a set of eight simple sequence repeat (SSR) primers, developed at the PGRU (Szewc-McFadden et al., 1995, 1996), to estimate overall levels of genetic diversity, assign unique genetic fingerprints to nearly all the accessions, and reveal meaningful molecular-based genetic relationships based on known pedigree information. We also uncovered previously unidentified

Table 1. Malus core species and hybrid accessions from the USDA-ARS at Geneva screened with SSR primers

PI	Accession
589727	*M. angustifolia* (Aiton) Michx.
589763	*M. angustifolia*
589222	*M.* × *arnoldiana* (Rehder) Sarg. ex Rehder 'Arnold Crab'
589253	*M.* × *atrosanguinea* (Spath) C.K. Schneid. 'Carmine Crab'
594099	*M.* × *asiatica* Nakai
589869	*M.* × *asiatica*
594107	*M.* × *asiatica*
322713	*M. baccata* (L.) Borkh. 'Mandshurica 2330'
588960	*M. baccata* 'Rockii'
437055	*M. baccata* 'Flexilis'
286599	*M. baccata*
594110	*M. baccata* 'Jackii'
589833	*M. baccata* 'Alexis'
589838	*M. baccata* 'Hansen's #2'
589976	*M. coronaria* (L.) Mill.
589996	*M. coronaria*
590020	*M. coronaria*
323617	*M. domestica* Borkh.
594106	*M. domestica*
588868	*M. florentina* (Zuccagni) C.K. Schneid.
589385	*M. florentina* 'Skopje P2'
589181	*M. floribunda* Siebold ex Van Houte 'Prima'
589741	*M. floribunda*
589827	*M. floribunda* '821'
589882	*M. doumeri* (Bois) A. Chev.
589933	*M. fusca* (Raf) C.K. Schneid.
589941	*M. fusca*
589975	*M. fusca*
594105	*M. fusca*
589246	*M. halliana* Koehne 'Parkman'
589972	*M. halliana*
594112	*M. halliana*
589879	*M. honanensis* Rehder
594113	*M. honanensis*
588760	*M. hupehensis* (Pamp.) Rehder
594098	*M. hupehensis*
589522	*M. hupehensis*
588804	*Malus* 'Kansas K14'
588870	*Malus* 'Dolgo'
588883	*Malus* 'Demir'
588992	*Malus* 'White Angel'
437057	*Malus* 'Roberts Crab'
590008	*M. ioensis* (A.W. Wood) Britton
590015	*M. ioensis*
596279	*M. ioensis* 'Texana'
588991	*M. ioensis* 'Bechtel Crab'
589999	*M. ioensis*

Table 1. Continued

PI	Accession
588944	*M. kansuensis* (Batalin) C.K. Schneid. 'Calva'
594097	*M. kansuensis*
589380	*M. kirghisorum* Al. Fed. & Fed.
590043	*M. kirghisorum*
588753	*M. mandshurica* (Maxim.) Kom.
594092	*M. micromalus* Makino
594093	*M. micromalus*
594096	*M. micromalus*
589753	*M. micromalus*
589955	*M. micromalus*
596278	*M. ombrophila* Hand.-Mazz.
596281	*M. ombrophila*
594095	*M. orientalis* Uglitzk.
594101	*M. orientalis*
589415	*M.* × *platycarpa* Rehder 'Hoopesii'
588933	*M. prattii* (Hemd.) C.K. Schneid.
590045	*M. prattii*
594102	*M. prunifolia* (Willd.) Borkh.
594103	*M. prunifolia* 'Inuringo'
589816	*M. prunifolia* '19651'
594109	*M. prunifolia* 'Microcarpa'
589832	*M. prunifolia* 'Xanthocarpa'
589930	*M. prunifolia* 'Naga'
589932	*M. prunifolia* 'MO-84'
588824	*Malus* 'Almey'
588866	*Malus* 'Kerr'
589478	*Malus* 'Novosibirski Sweet'
590069	*Malus* 'E7-47'
590070	*Malus* 'E7-54'
590071	*Malus* 'E29-56'
590072	*Malus* 'E31-10'
589570	*Malus* 'E36-7'
589571	*Malus* 'E11-24'
589572	*Malus* 'E14-32'
589829	*Malus* 'PRI 333-9'
589775	*Malus* 'PRI 2382-1'
589776	*Malus* 'PRI 1316-1'
589777	*Malus* 'PRI 1918-1'
589780	*Malus* 'PRI 384-1'
589785	*Malus* 'PRI 1346-2'
589786	*Malus* 'PRI 77-1'
589789	*Malus* 'PRI 1744-1'
589790	*Malus* 'PRI 1484-1'
589791	*Malus* 'PRI 1279-9'
589792	*Malus* 'PRI 1850-4'
589794	*Malus* 'PRI 1754-2'
590079	*Malus* 'PRI 1312-6'
590085	*Malus* 'PRI 1176-1'
589795	*Malus* 'PRI 2482-100'
589819	*Malus* 'PRI 2050-2'

Table 1. Continued

PI	Accession
589807	*Malus* 'PRI 1773-6'
589812	*Malus* 'PRI 2377-1'
589946	*Malus* 'PRI 1732-2'
589420	*M.* × *hartwigii* Koehne
589820	*Malus* 'Prairie Fire'
589824	*Malus* 'Jonsib Crab'
589958	*Malus* 'MA #4'
589421	*Malus* 'Rockii'
589805	*Malus* 'Co-op 15'
589170	*Malus* 'Brevipes'
483254	*Malus* 'Dawsoniana' Rehder
588757	*Malus* 'Hartwigii' Koehne
588825	*M.* × *robusta* (Carriere) Rehder 'Robusta 5'
589003	*M.* × *robusta* 'Korea'
589383	*M.* × *robusta* 'Persicifolia'
588761	*M. sargentii* Rehder
589405	*M. sargentii*
588959	*Malus* × *magdeburgensis* Hartwig
589835	*Malus* 'Russian Seedling. #12740-7A'
594094	*M. sieboldii* (Regel) Rehder
589749	*M. sieboldii*
594104	*M. sieversii* (Ledeb.) M. Roem.
596282	*M. sieversii*
596280	*M. sieversii*
596283	*M. sieversii*
589390	*M. sikkimensis* (Wenz.) Koehne ex C.K. Schneid.
589834	*M. sikkimensis*
589391	*M.* × *soulardii* (L.H. Bailey) Britton
588893	*M. spectabilis* (Afton) Borkh. 'Plena'
594100	*M. spectabilis*
588922	*M.* × *sublobata* (Dippel) Rehder 'Yellow Autumn' Crab'
369855	*M. sylvestris* Mill.
589382	*M. sylvestris*
377590	*M. sylvestris*
588920	*M. toringoides* (Rehder) Hughes 'Cut-Leaved Crab'
588930	*M. toringoides* 'Macrocarpa'
589393	*M. toringoides*
589384	*M. transitoria* (Batalin) C.K. Schneid.
589422	*M. transitoria*
589397	*M. trilobata* (Poir.) C.K. Schneid.
589395	*M. tschonoskii* (Maxim.) C.K. Schneid.
589399	*M. yunnanensis* (Franch.) C.K. Schneid.
271831	*M. yunnanensis* 'Vilmorin'
589758	*M. yunnanensis* 'Veichii'
589840	*M. zumi* (Matsum.) Rehder 'Calocarpa'

mislabeled accessions in the collection. In this study, we extend the previously reported molecular characterization to the remaining 142 members of the *Malus* core collection, which includes all the *Malus* species and a number of their hybrids. In addition we investigate whether the eight SSR primers described previously would amplify products in SSR reactions with *Malus* species and hybrids genotypes representative of all accessions curated at the PGRU.

Materials and methods

Characterization of the Malus *species and hybrids collection*

Genomic DNA was extracted from leaves of the 142 *Malus* species and hybrid accessions (Table 1) using the DNA extraction protocol described by Lamboy & Alpha (1998). PCR amplifications were conducted on the genomic DNA with three multiplexed primer sets comprised of the eight microsatellite primers described by Hokanson et al. (1998). Methodologies and protocols utilized in this project were identical to those described previously. The primers have been analyzed in several segregating apple mapping populations (Hemmat et al., 1998).

Allele frequencies, alleles per locus, direct count heterozygosity, polymorphic information content (PIC) (Röder et al., 1995), discrimination power (Jones, 1972; Kloosterman et al., 1993), and Nei's genetic identities (Nei, 1972) were calculated using the computer program 'SSRS' written by Lamboy using the Microsoft Fortran Powerstation for IBM-compatible PCs running Windows. Effective alleles per locus (A_{ep}) were calculated according to Weir (1989) with the formula $1/(1–H_{ep})$, where H_{ep}, the genetic diversity per locus, is equal to $1 - \Sigma p_i^2$ and p_i^2 is equal to the frequency of the ith allele at the locus. Direct count heterozygosities were calculated as the number of genotypes which were heterozygous at a given locus divided by the total number of genotypes scored at that locus. Polymorphic information contents (PIC) were calculated with the following formula, $1 - \Sigma_{i=1}^{n} p_i^2$, where p_i equals the frequency of the ith allele. The discrimination power at a locus, which provides an estimate of the probability that two randomly sampled accessions in the study would be differentiated by their allelic profiles, was obtained for both the sample under investigation and an infinitely large theoretical population with the same genotype

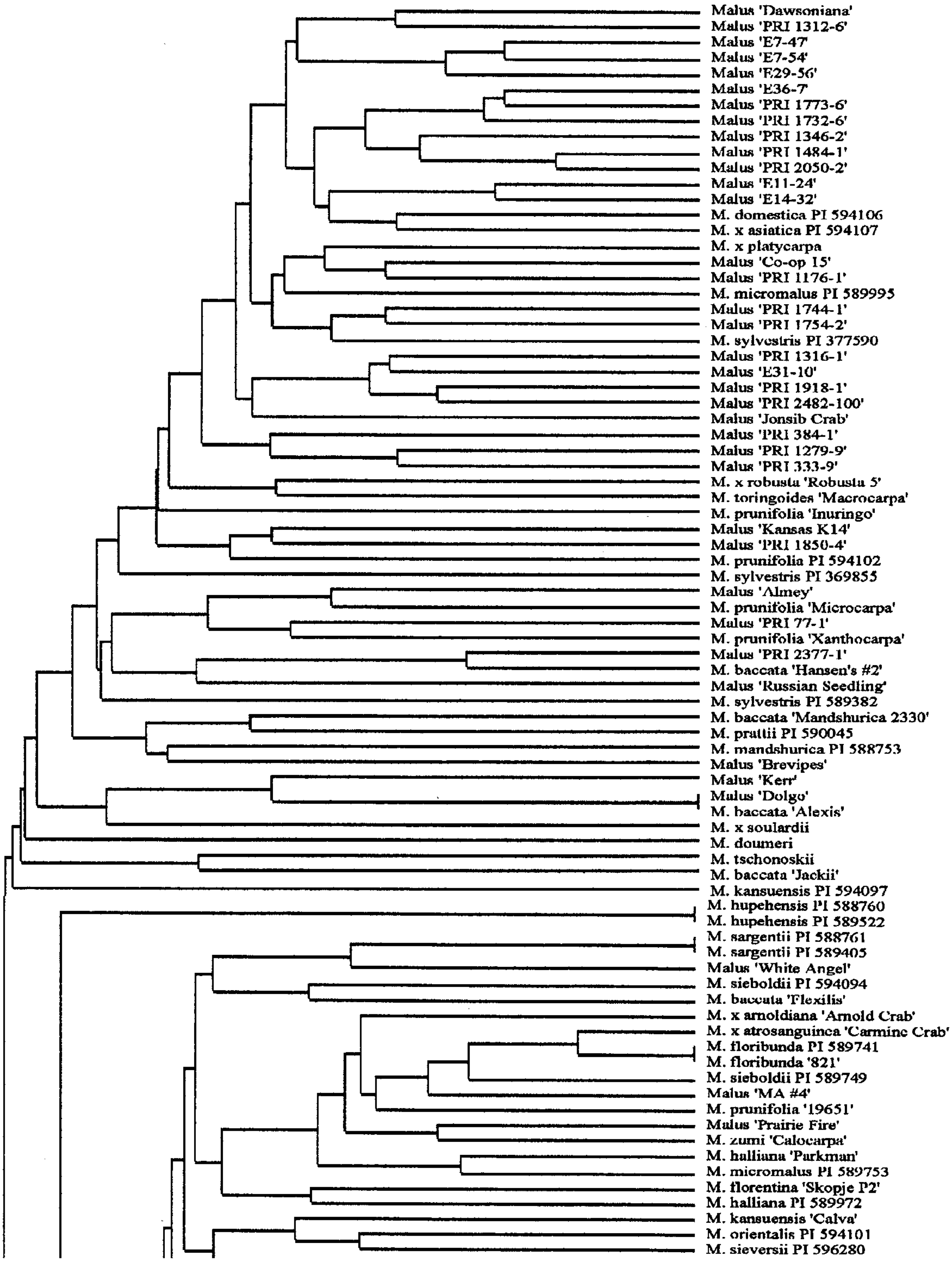

Figure 1. (Cont. on pg. 286)

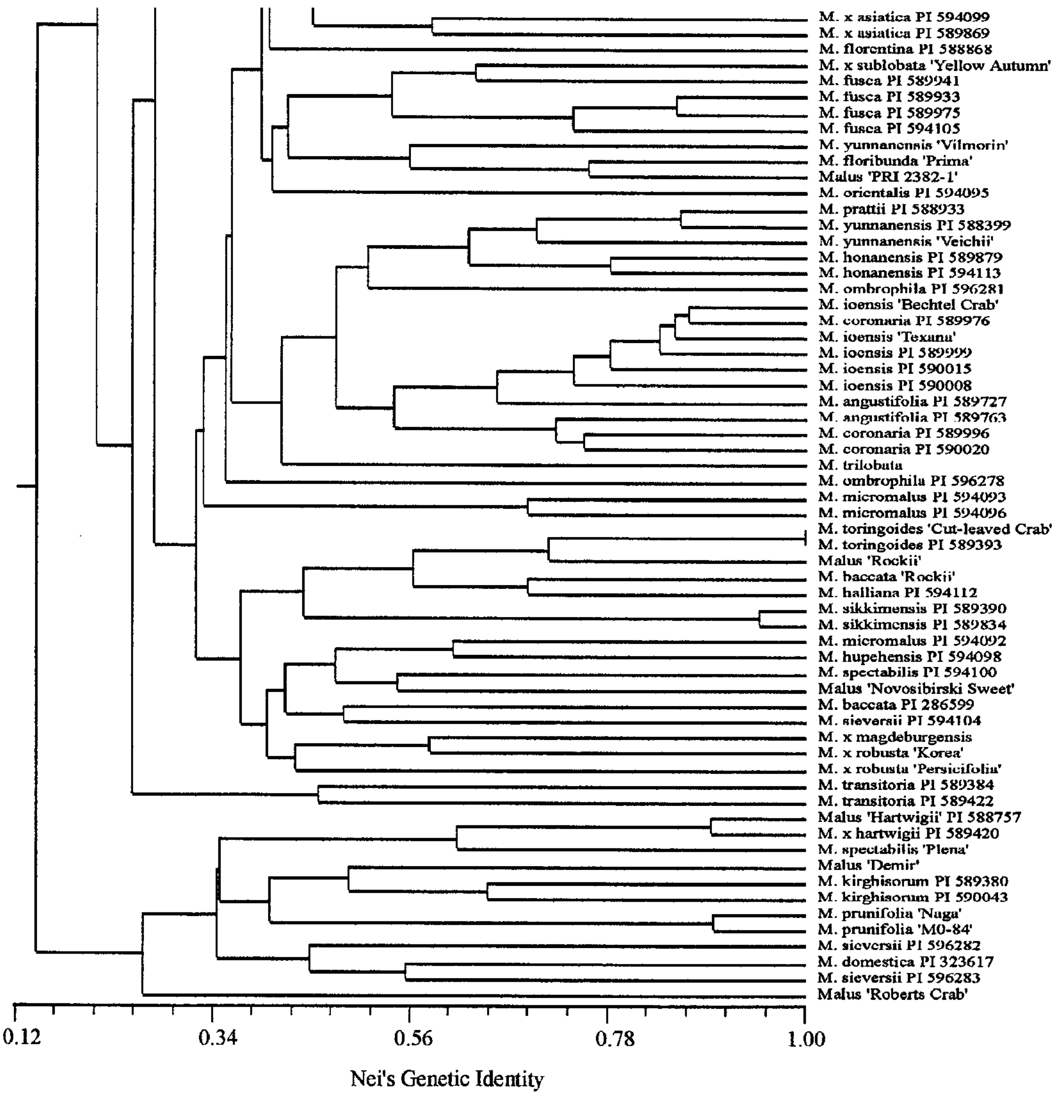

Figure 1. Unweighted pair-group method analysis (UPGMA) phenogram for the 142 *Malus* species and hybrid accessions evaluated in this study. The phenogram was produced using an UPGMA cluster analysis of Nei's genetic identities between the accessions.

frequencies found in the sample population. The value was calculated with the formula $1 - \Sigma(p_i)^2$, where p_i represents the frequency of each genotype (Kloosterman et al., 1993). As in our previous analysis, accessions that showed only one fragment at a locus were considered to be homozygous for that fragment. If the accession were actually heterozygous for the fragment and a null allele, the results reported would be an underestimate of the levels of heterozygosity and gene diversity in the collection. Accessions were scored as nulls at a locus when after multiple runs, no product was amplified at the locus.

Genetic relationships among the 142 accessions in this study were investigated using an unweighted pair-group method (UPGMA) cluster analysis of Nei's genetic identities for the accessions (Sneath & Sokal, 1973). The analysis and a phenogram (Figure 1) were computed with the program NTSYS-pc, ver. 2.01 (Rohlf, 1998).

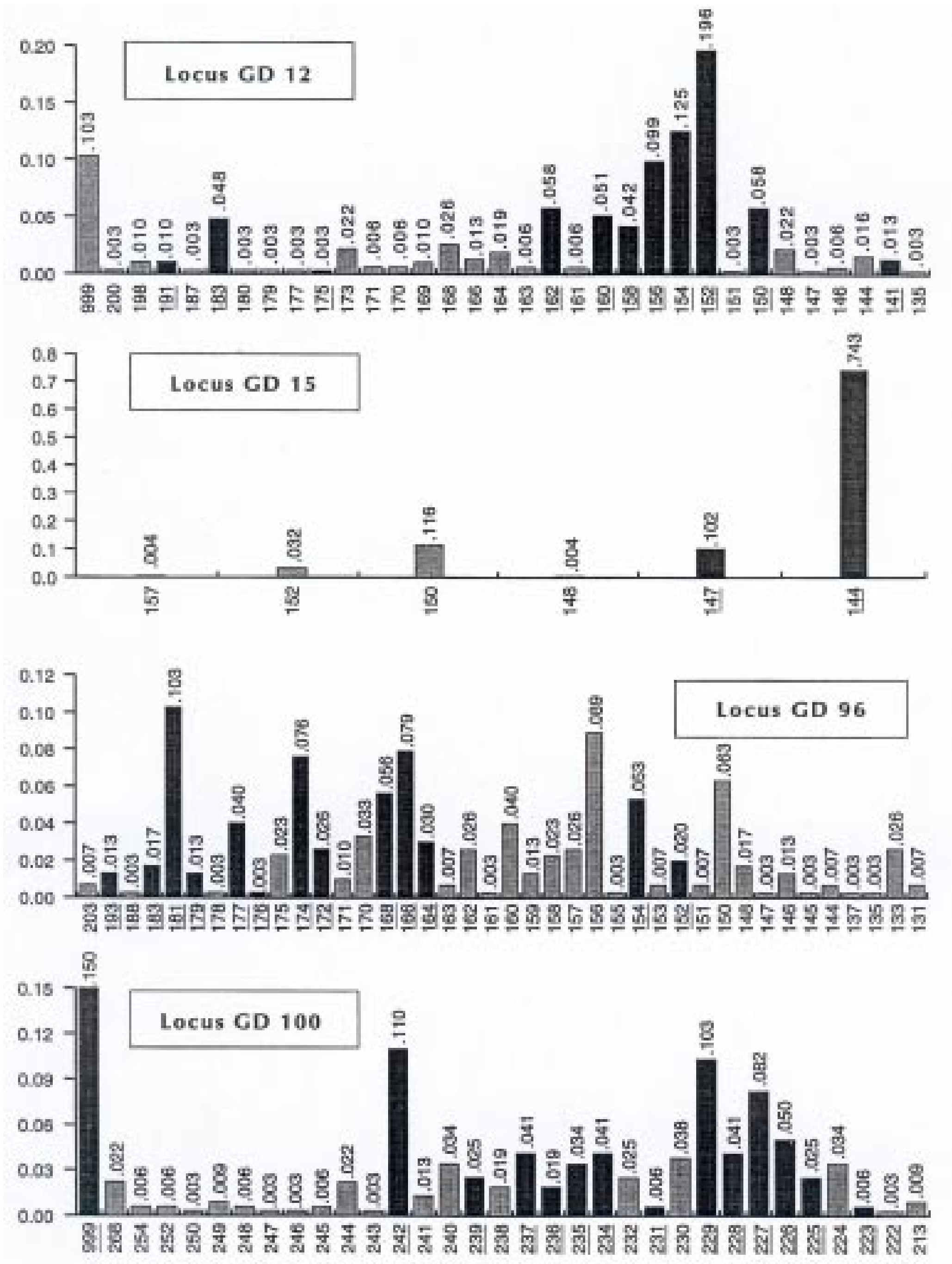

Figure 2. Eight histograms depicting the alleles that occurred at each locus, listed by base-pair size (abscissa) with 999 representing null alleles, and the frequency at which each allele occurred (ordinate) in this collection of 142 *Malus* species and hybrid accessions. Underlined size values and corresponding darkened bars represent alleles noted in previous study of 66 *Malus* × *domestica* Borkh. accessions.

Table 2. SSR primer product characterization

Locus	Expected product size (bp)	Range of product sizes (bp)	Number putative alleles	A_{ep}[z]	Direct count heterozygosity	Polymorphic information content	Discrimination power
GD 12	192	135–200	33 (*12*)[y]	10.99	0.746 (*0.758*)[y]	0.909	0.982
GD 15	144	144–157	6 (*2*)	1.73	0.289 (*0.015*)	0.423	0.598
GD 96	173	131–203	40 (*15*)	19.40	0.866 (*0.909*)	0.948	0.993
GD 100	227	213–268	33 (*14*)	14.59	0.697 (*0.879*)	0.931	0.987
GD 103	108	90–133	18 (*13*)	3.07	0.113 (*0.333*)	0.675	0.694
GD 142	143	123–189	23 (*13*)	10.43	0.803 (*0.909*)	0.904	0.984
GD 147	138	114–170	25 (*15*)	12.50	0.761 (*0.848*)	0.920	0.986
GD 162	234	189–252	33 (*13*)	11.10	0.711 (*0.894*)	0.910	0.978
		$\bar{x}$	26.4	10.48	0.623	0.828	1.000[x]

[z] Effective alleles per locus.
[y] Italicized, parenthetical putative alleles per locus and direct count heterozygosities per locus values from 66 *Malus* × *domestica* accessions characterized in Hokanson et al., 1998.
[x] Value is the total discrimination power for all loci.

Results

Genetic diversity

All eight primer pairs generated multiple fragments (alleles) when amplified in SSR reactions with genomic DNA from each of these 142 *Malus* accessions. The number of alleles per locus in this study ranged from six for GD 15 to 40 for GD 96, with a mean value over all loci of 26.4 (Table 2). The relative number of alleles per individual locus found in this study of species and hybrids was similar to that found in our previous study of *M.* × *domestica*. GD 96 had the most alleles in both the cultivated (15) and species (40) subsets, respectively. Interestingly, locus GD 147 ranked first with GD 96 (15 alleles) in the *M.* × *domestica* subset, but fell to fifth position (25 alleles) in the species subset, while GD 12 went from seventh (12 alleles) in the *M.* × *domestica* group to second in the species group (Table 2).

One hundred and twelve null alleles were detected in the 1,136 possible accession-by-loci combinations in this study. Nulls were detected in 76 of the accessions examined, with 68% of the nulls occurring at locus GD 103. Only a single null was detected for the majority of the accessions; however, more than half of the multiple null genotypes (13) were detected in the North American *Malus* complex.

Frequencies for individual alleles at all loci were generally low, with only four alleles having values greater than twenty percent at a locus (Figure 2). Direct count heterozygosities for individual loci ranged from 0.866 at GD 96 to 0.113 at GD 103 and was 0.623 for all loci in the study (Table 2). Genetic diversity or polymorphic information content (PIC) values per locus ranged from 0.423 at GD 15 to 0.948 at GD 96, with an average PIC value for all loci of 0.828 (Table 2). Polymorphic information content values did not always correspond with the level of heterozygosity at a given locus. For example, even though the direct count heterozygosity at GD 103 was the lowest in this study (0.113), the PIC value at the locus was 0.675, higher than that detected at GD 15, which had a direct count heterozygosity of 0.289 and a PIC value of 0.423. This is due to the fact that the PIC statistic is an estimate analogous to the expected heterozygosity statistic based on Hardy-Weinberg expectations. In contrast, the direct count heterozygosity statistic is an actual count of heterozygous genotypes.

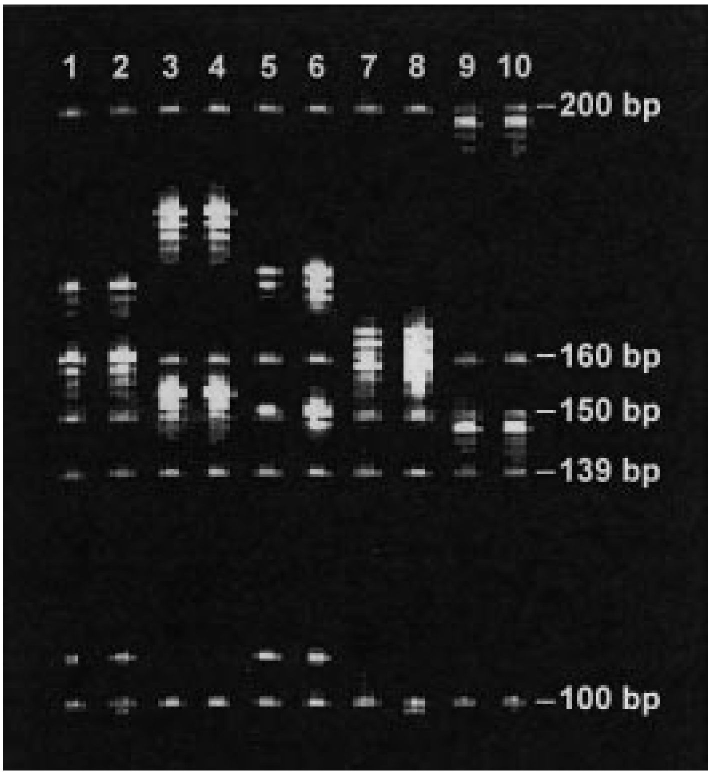

Figure 3. SSR gel image depicting the reaction products from PCR amplifications of genomic DNA from 10 *Malus* species and hybrid accessions amplified with the GD 12 SSR primer pair. Labeled bands are the 350-Tamara internal lane standards sized in base pairs. Lanes 1 and 2 contain amplification products from the two indistinguishable *M. hupehensis* accessions, PI 588760 and 589522, respectively. Lanes 3 and 4 contain products from *M.* 'Dolgo' and *M. baccata* 'Alexis', PI 588870 and 589833, respectively. Lanes 5 and 6 contain products from the two indistinguishable *M. floribunda* accessions, PI 589741 and 589827, respectively. Lanes 7 and 8 contain amplification products from the two indistinguishable *M. sargentii* accessions, PI 588761 and 589405, respectively. Lanes 9 and 10 contain products from the two indistinguishable *M. toringoides* accessions, PI 588920 and 589393, respectively.

Genetic identity

The eight SSR primer pairs unambiguously differentiated all but five pairs of accessions in the collection of 142 *Malus* species and hybrids evaluated. Eighty-five percent of the accessions differed for at least seven of the eight primer pairs. The discrimination power at a locus in this study ranged from 0.598 at GD 15 to 0.993 for GD 96. The overall discrimination power of all loci in the study was effectively equal to 1.00 (Table 2), suggesting that all genetically unique accessions in the study could be identified by the respective genetic fingerprint generated by the eight primer pairs.

The five undifferentiated genotype pairs were: *M. hupehensis* (Pampan.) Rehder PI 588760 and *M. hupehensis* PI 589522, *M.* 'Dolgo' PI 588870 and *M. baccata* 'Alexis' PI 589833, *M. floribunda* PI 589741 and *M. floribunda* '821' PI 589827, *M. sargentii* Rehder PI 588761 and *M. sargentii* PI 589405 and *M. toringoides* (Rehder) Hughes 'Cut-leaved crab' PI 588920 and *M. toringoides* PI 589393 (Figure 3). In addition, one pair of accessions, *M. prunifolia* 'Naga' and *M. prunifolia* 'MO-84', were differentiated by only two primer pairs, GD 12 and GD 142.

Genetic relatedness

The UPGMA cluster analysis utilized in this study produced three nearly identical trees. The trees differed only in the ordering of twelve accessions and because none provided a clearer picture of genetic relationships among the accessions, we present one tree to represent the analysis (Figure 1). Unlike our previous report of 66 *M. domestica* accessions, the majority of the accessions in this study did not cluster in any groups that were consistent with known pedigree information and/or geographic origins of the accessions. For example, this collection contains six *M. prunifolia* accessions of which only two, *M. prunifolia* PI 589930 and *M. prunifolia* PI 589932 cluster together in pairs, with the rest found scattered throughout the phenogram. Even more dispersion is seen among the accessions of *M. baccata, M. micromalus*, and *M. sieversii*.

Two cohesive groups did form in the analysis that were somewhat consistent with geographic origin. One grouping consisted of three of the four accessions of a North American species, *M. fusca* (Raf.) L. Schneider, which were collected in 1988 from their native habitat along the North American Pacific coast from Alaska to northern California. This group clustered more distantly with the other *M. fusca*, PI 589941 accession; however that accession was clustered with the *M.* × *sublobata* 'Yellow Autumn' accession PI 588922, which has no obvious relation with the *M. fusca* accessions. The second grouping, consisting of ten accessions, represents the three other North American *Malus* species: *M. ioensis* (Alph. Wood) Britton, *M. coronaria* (L.) Miller, and *M. angustifolia* (Aiton) Michaux. All of these accessions were collected within their native range and all except two of the *M. ioensis* accessions, PI 588991 and 596279, were collected relatively recently, between 1985 and 1988.

Discussion

The eight SSR primer pairs we used in this study generated multiple alleles when amplified in SSR reactions across the complete range of *Malus* species and hybrids that are curated at the USDA-ARS repository in Geneva. The fact that the primers amplify products across the genus *Malus* will allow systematic and uniform comparisons of genetic identity and diversity data between cultivated apples and related *Malus* species and hybrids to be made. The high levels of variability and reproducibility associated with SSR markers allow them to serve as anchor markers between different genetic maps within a crop (Beckmann & Soller, 1990; Cregan et al., 1999). The eventual positioning of these SSR loci on maps resulting from diverse mapping populations will facilitate the identification and movement of critical genes conferring biotic and abiotic resistances and tolerances as well as important horticultural traits found within diverse *Malus* germplasm in a manner similar to that described for tomato and rice (Tanksley & Nelson, 1996; Xiao et al., 1996). Currently, several *Malus* mapping projects include the positioning of SSR loci on diverse apple genetic maps (Gianfranceschi et al., 1998; King, 1996; Maliepaard et al., 1998; Hemmat et al., 1998).

A higher number of alleles per locus were detected among this group of 142 *Malus* species and hybrids than among the 66 cultivated types described in a similar survey (Hokanson et al., 1998). This is not unexpected given that most modern cultivars result from a restricted number of founding clones ('Cox's Orange Pippin', 'Golden Delicious', 'Red Delicious', 'Jonathan', and 'McIntosh'), which should result in a concomitant decrease in genetic diversity (Noiton & Alspach, 1996). However, in contrast to the comparison of alleles per locus, the domesticated apples had higher levels of heterozygosity per locus than the *Malus* species and hybrids except at the GD 15 locus. Lamboy & Alpha (1998) also found higher levels of heterozygosity in domesticated *Vitis* cultivars in comparison to *Vitis* species. They speculated that in the process of selecting cultivars, improvements in horticultural characters may be conferred by higher levels of heterozygosity. For several highly heterozygous horticultural crops, including apple, the deleterious effects of inbreeding can be seen in only a few of cycles of inbreeding (Janick et al., 1996). In addition, the existence of a self-incompatibility system in apple necessitates crossing compatible types that would result in higher levels of heterozygosity.

An alternative explanation for the higher levels of heterozygosity detected in domesticates than in related species and interspecific hybrids in these studies might be the phenomenon referred to as 'ascertainment bias' (Ellegren et al., 1995). In a study of swallows (*Hirundo rustica*) and closely related species of the genus *Hirundinidae* with microsatellites developed in *H. rustica*, referred to as the 'focal species', the microsatellites were found to be longer in the focal species and they detected more diversity within the focal species than in the closely related species. In both the *Vitis* and *Malus* examples referred to above, the SSR primers were developed from a domesticated cultivar. Ascertainment bias could explain the increased levels of heterozygosity seen in the domesticated collections as compared to the related species and hybrids.

In this study 112 null alleles were detected among the 1,136 possible genotype by loci combinations, approximately a 10% frequency. In contrast, eight nulls were detected in our previous study of 66 *M. domestica* accessions, a frequency of approximately two percent. The increased frequency of the nulls in the present study may be another effect of the ascertainment bias phenomenon. The majority of the nulls (68%) occur at a single locus, GD 103, with the majority of the accessions displaying a single null genotype. Interestingly, the majority of the multiple null genotypes occur in the North American species complex; *M. angustifolia, M. coronaria, M. fusca*, and *M. ioensis*. These accessions constituted the only clusters in the genetic relatedness analysis that made sense in light of the geographic origins or known pedigree information regarding the genotypes (Figure 1).

The results from this study coupled with results from our previous study of 66 *M.* $\times$ *domestica* accessions demonstrate the effectiveness of SSRs for providing unique genetic identities for each accession in a germplasm collection. The high discrimination power across all loci in the two studies, effectively equal to one, resulted in the unambiguous differentiation of all accessions in the collection. When any two genotypes in the collection are identical at all loci, trueness to type for the genotype immediately comes into question. In our study of 66 domesticated apples, one accession was found to be mislabeled, while another ('Chihuahua Gold') was found to be genetically indistinguishable from 'Golden Delicious'.

The identical genotypes detected in the current study were observed in the field and found to display identical morphological traits. Subsequently, for the two *M. hupehensis* accessions, PI 589522 was saved and PI 588760 was discarded. *Malus* 'Dolgo' was saved while *M. baccata* 'Alexis' was discarded. For the two *M. floribunda* accessions, '821' was saved and PI 589741 was discarded. Among the two *M. sargentii* accessions, PI 588761 was saved while PI 589405 was discarded. The *M. toringoides* accession PI 589393 was saved, while the PI 588920 was discarded. Detecting these identical genotypes in the current study raises a new, different concern. Previously it was suggested that the USDA-ARS *Malus* collection was seriously under represented with regards to several species (Hokanson et al., 1997; Forsline & Way, 1993). In this study we found a number of accessions within these species to be duplicates. This suggests that the under representation of species material in the PGRU may be more critical than originally envisioned. The two identical *M. hupehensis* accessions represent nearly ten percent of the repository holdings for that species. Similarly, the duplicated accessions of *M. floribunda, M. sargentii* and *M. toringoides* represent approximately fourteen, ten, and forty percent of the total holdings for the respective species. Additionally, the lack of passport information for some of the species accessions in the collection raises concerns regarding trueness-to-type. Many accessions, including some of the duplicates considered herein, were acquired from other sources and are in effect several generations removed from the original point of collection from their native habitat. *M. floribunda* '821' was grown in Illinois from seed acquired from the Arnold Arboretum in 1908 and the passport data does not make it clear whether the seed was collected from trees growing in Massachusetts or from native stands in Japan.

Sax (1959) reported facultative apomictic reproduction in *M. hupehensis, M. sargentii* and *M. toringoides*. Thus, seed collections of these species may contain many genetically identical progeny with only a few hybrid seed. The World Conservation Union has listed *M. hupehensis* as a globally endangered species (Walter & Gillett, 1998). In addition to being apomictic, the species *M. sargentii* is only represented by a few clones worldwide (Way et al., 1991). The question of whether wild populations of the species still exist is a matter of debate. The discovery that two assumed distinct genotypes of this potentially rare species are actually genetically identical erodes the world's presumed genetic base of the species substantially.

Although the genetic relatedness analysis did produce two meaningful clusters based on pedigree and geographic origins of the accessions, the phenogram produced in this study was not as meaningful as that produced in our previous study of 66 *M. domestica* accessions (Hokanson et al., 1998). In a RAPD analysis of 18 *Malus* species and 27 apple cultivars, Dunemann et al. (1994) report somewhat similar results. Cultivars with 'fully identified lineage' grouped in a manner consistent with genetic origin and although cultivars with uncertain origins did not group as well, the analysis did reveal commonalities in ancestry and shed light on long standing pedigree questions. The dendrogram produced for the wild species was more problematic but gave results that were principally in accordance with accepted phylogenies. However, not all the primary species were included in the study and in most cases, only single representatives of a species was used. The authors suggest the RAPD markers have the potential to complement 'classical' taxonomic studies, however larger numbers of and/or different molecular markers and statistical methods should be utilized.

As suggested by Dunemann et al. (1994), the difficulty in producing a meaningful genetic relatedness phenogram in the present study could be due to the inappropriateness of the SSR markers for resolving relatedness at this higher (interspecific) taxonomic level. The problem could also be due in part to the makeup of the collection investigated in this study. When microsatellites first became a viable option for plant genetics research, the huge number of potentially available microsatellites and their elevated mutation rates rendered them primary candidates for investigating genetic relatedness at the intra- and interspecific level. Although SSRs have been widely and successfully used at the intraspecific level, their utility at the interspecific level has been less than expected.

Several hypotheses for the failings of microsatellites at the interspecific level have been suggested, including: restrictions to divergence in the repeat sequences, asymmetries in the mutation process in the repeat regions, and the degradation of the microsatellite regions over time (Goldstein & Pollock, 1997). As noted earlier, another possible factor might be ascertainment bias (Ellegren et al., 1995). Since length and variability in microsatellites are correlated (Weber 1990; Garza et al., 1995), less variability was witnessed at the SSR locus in the related species than in the focal species. This bias requires the sequencing of SSR alleles to insure that the microsatellite loci employed to assess interspecific relatedness in such studies are consistent across the species being investigated (Goldstein & Pollock, 1997).

Aside from the limitations of the markers utilized in this assessment, the particular plant collection under investigation poses another set of problems. Some of the species accessions in the collection, including all the *M. micromalus* accessions, have inadequate passport data. Some of the accessions, including *M. floribunda* '821' and two of the *M. baccata* accessions, were collected as seed. In this highly outcrossing, highly heterozygous genus, the trueness to type of such seedlings might be questioned. There is also some temporal variation to the collection. Some of the accessions were collected in the late 1800's in their respective centers of origin, while later accessions of the same species were collected in the same relative locations late in the 20th century. Depending on the age cohort from which the respective accessions arose and the size and genetic make-up of the surrounding *Malus* populations at the time, the genetic constitution of the accessions could be considerably different. Around the world, wild populations of *Malus* are being reduced in size and eliminated due to human activities (Hokanson et al., 1997; Way et al., 1991).

In contrast, the accessions that constitute the North American species groups were generally collected within a very short time frame in the late 1980's from within their known range of distribution. Each of these accessions was entered into the collection with comprehensive passport data. Interestingly, these accessions clustered in groups that were consistent with accepted taxonomic treatments and geographic origin. This finding suggests that some of the problems we encountered in this genetic relatedness analysis may be due in part to the makeup of the collection itself. Although a genetic relatedness analysis of 142 genotypes was a large undertaking, the small number of genotypes constituting each species group was suboptimal and a larger number of accessions of each species should be included. Unfortunately, germplasm collections do not lend themselves to such optimization. Older accessions may be inadequately characterized and questionable with regard to trueness to type. Additional accessions may never be available. In such cases, inconclusive molecular data will necessarily need to be combined with all available morphological and horticultural data to make decisions regarding the utility of such germplasm.

Despite the inadequacy of SSRs in resolving higher taxonomic relationships, the markers have proven to be quite robust for many germplasm management applications. Eight SSR markers provided reliable, unique genetic fingerprints that allowed for unambiguous differentiation of all accessions in the repository collection while simultaneously allowing for measures of genetic diversity. The capacity of the markers to amplify products across the complete range of *Malus* species and hybrids has opened the door for efforts to develop genetic maps in widely divergent mapping populations in different labs around the world, a process that is already underway.

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